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Inorganic Carbon Uptake during Photosynthesis[1]

II. UPTAKE BY ISOLATED *ASPARAGUS* MESOPHYLL CELLS DURING ISOTOPIC DISEQUILIBRIUM

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ABSTRACT

The species of inorganic carbon (CO_2 or HCO_3^-) taken up a a source of substrate for photosynthetic fixation by isolated *Asparagus sprengeri* mesophyll cells is investigated. Discrimination between CO_2 or HCO_3^- transport, during steady state photosynthesis, is achieved by monitoring the changes (by ^{14}C fixation) which occur in the specific activity of the intracellular pool of inorganic carbon when the inorganic carbon present in the suspending medium is in a state of isotopic disequilibrium. Quantitative comparisons between theoretical (CO_2 or HCO_3^- transport) and experimental time-courses of ^{14}C incorporation, over the pH range of 5.2 to 7.5, indicate that the specific activity of extracellular CO_2, rather than HCO_3^-, is the appropriate predictor of the intracellular specific activity. It is concluded, therefore, that CO_2 is the major source of exogenous inorganic carbon taken up by *Asparagus* cells. However, at high pH (8.5), a component of net DIC uptake may be attributable to HCO_3^- transport, as the incorporation of ^{14}C during isotopic disequilibrium exceeds the maximum possible incorporation predicted on the basis of CO_2 uptake alone. The contribution of HCO_3^- to net inorganic carbon uptake (pH 8.5) is variable, ranging from 5 to 16%, but is independent of the extracellular HCO_3^- concentration. The evidence for direct HCO_3^- transport is subject to alternative explanations and must, therefore, be regarded as equivocal. Nonlinear regression analysis of the rate of ^{14}C incorporation as a function of time indicates the presence of a small extracellular resistance to the diffusion of CO_2, which is partially alleviated by a high extracellular concentration of HCO_3^-.

It is now apparent that there are a number of specialized mechanisms by which plant cells acquire DIC[4] from their surroundings for photosynthetic fixation (15) and that the various means of acquisition dramatically affect the efficiency of photosynthetic C assimilation (3–5, 7, 13). In cyanobacteria, for example, HCO_3^- is actively transported across the plasmalemma and accumulated within the cells (2, 13, 16). This process serves to increase the intracellular CO_2 concentration well above the ambient level, resulting in the suppression of photorespiration and enhancement of photosynthesis.

Recent studies with mesophyll cells (19, 20) and protoplasts isolated from the leaves of C_3 terrestrial plants suggest that they may also be capable of transporting HCO_3^- across the plasmalemma. These results, however, conflict with other reports which indicate that the diffusive flux of CO_2 across the plasmalemma is the sole means of DIC acquisition (7, 9, 12, 18). Since the uptake of HCO_3^- by C_3 mesophyll cells may serve to concentrate intracellular CO_2 to some extent and/or to enhance the flow of C from the environment to the site of photosynthetic carboxylation, it is important to determine whether or not HCO_3^- transport occurs.

In this paper, we assess the contributions of exogenous CO_2 and HCO_3^- to the intracellular supply of DIC during steady state *Asparagus* cell photosynthesis and investigate the possibility and significance of carrier-mediated HCO_3^- transport. For this purpose, we have employed an isotopic disequilibrium technique (2, 8, 14) which permits the quantitative separation of the maximum contribution of CO_2 uptake to photosynthesis from that which may occur from the uptake of HCO_3^-. A detailed account of the theory (8, 14) and calculations (8) of this procedure have been presented.

MATERIALS AND METHODS

Mesophyll cells were mechanically isolated (5, 6) from cladophylls of greenhouse-grown *Asparagus sprengeri* Regel plants. Prior to experiments, the cells were stored in 50 mM Hepes buffer (pH 7.2) for 12 to 16 h in the dark at 10°C. No significant decline in the rate of photosynthesis occurred as a result of the storage (5).

Experiments were conducted at 25°C, and illumination was provided by two 150 W photocreseta lamps positioned to give 550 μmol (photons)$\cdot m^{-2}\cdot s^{-1}$ (400–700 nm) at the back surface of the reaction vessel. In experiments in which the pH was varied, the following 50 mM buffers were employed: Mes, 5.2 to 6.5; Hepes, 7.5; Bicine, 8.5. The pH of the cell suspension was measured after each experiment and this value was used in subsequent calculations. The pH did not change more than 0.03 units.

Photosynthesis. The rate of photosynthesis was measured as O_2-evolution using a Clark-type electrode (Hansatech, Kings Lynn, Norfolk, U.K.) and was routinely used as a measure of carbon assimilation. This approach is justified as independent experiments indicated that, under the conditions used here, the ratio of O_2-evolution to CO_2-fixation is 1:1 (not shown). The Chl concentration of cell suspensions was determined by the method of Arnon (1).

Isotopic Disequilibrium Procedure. *Asparagus* cells were washed twice by centrifugation (100*g*, 10 min; 15,000*g*, 6 s), resuspended in an appropriate buffered solution, and preincubated for 5 to 7 min in the light at the reaction pH. The cells were again pelleted by centrifugation (15,000*g*, 6 s), resuspended in 2.9 ml of fresh buffer (15–25 μg Chl$\cdot$ml^{-1}), and the suspension

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[4] Abbreviations: DIC, dissolved inorganic carbon; CA, carbonic anhydrase; dpm, disintegrations per minute; SA, specific activity (superscript: *B*, $H^{14}CO_3^-$ addition; *C*, $^{14}CO_2$ addition; subscript i, intracellular).

was placed in the O_2-electrode chamber. An aliquot was withdrawn for Chl analysis. Photosynthesis was initiated by the addition of $NaHCO_3$, and the cells were allowed to reach a steady state rate of O_2 evolution (3–5 min). Subsequently, the cap of the O_2-electrode chamber was removed, and the isotopic disequilibrium experiment was commenced by the addition of a μl quantity of $H^{14}CO_3^-$ or $^{14}CO_2$ to the rapidly stirred cell suspension. Following 6 s of mixing, the cell suspension (2.2 ml) was drawn into the tip of a transparent Eppendorf repeater pipette and positioned in front of a water-shielded lamp (550 μmol·m^{-2}·s^{-1}). The reaction was stopped at timed intervals (3 or 6 s) by injecting a 100 μl aliquot of suspension into 400 μl of terminating solution (water:acetic acid:methanol, 50:40;10, v/v/v), contained in a scintillation vial. The contents of the vial were evaporated to dryness, the residue suspended in 250 μl of distilled H_2O, and the incorporation of ^{14}C into acid-stable products was determined by scintillation counting (Packard Tricarb 300 CD) following the addition of 6 ml of scintillation fluid (ACS, Amersham, Toronto, Canada). The final two samples were injected into vials containing 400 μl of 0.2 M NaOH, and the total radioactivity of the suspension was measured by scintillation counting.

$NaH^{14}CO_3$ (54–57.8 μCi·μmol^{-1}) was supplied by Amersham (Toronto, Canada) and was used as received (pH 9.8). The $^{14}CO_2$ was generated by injecting $NaH^{14}CO_3$ into CO_2-free acetate buffer (5 mM, pH 4) contained in a sealed vessel. To correct for the continuous loss of $^{14}CO_2$ to the head-space, the solution was routinely assayed for radioactivity immediately prior to its use. The appropriate μl quantity of $^{14}CO_2$, to be added to the cell suspension, was calculated from the result of the assay.

Measurement of DIC and SA_{DIC}. The DIC concentrations of stock $NaHCO_3$ solutions and air-equilibrated buffers were measured using a sensitive gas-chromatographic technique (4). The specific activity of the bulk DIC (SA_{DIC}), in units of μCi·μmol^{-1}, was calculated from the results of total radioactivity assays (μCi) and DIC measurements (μmol). Corrections to SA_{DIC} were made to compensate for photosynthetic DIC consumption, which occurred prior to the addition of ^{14}C, and for the small quantity of DIC added as ^{14}C.

Isotopic Disequilibrium: Theory and Calculations. When $H^{14}CO_3^-$ or $^{14}CO_2$ is added to an aqueous system containing a relative excess of unlabeled DIC, such that neither the pH nor bulk DIC concentration is altered, then the various DIC species will be in a state of isotopic disequilibrium (14). Initially, all of the radiocarbon is present in one chemical form while the bulk, unlabeled DIC species are present in equilibrium proportions (14). Thus the initial value of SA_{CO2} and SA_{HCO3}^- are distinctly different. However, the value of SA_{CO2} and SA_{HCO3}^- exponentially approach a common value, SA_{DIC}, as the system achieves isotopic equilibrium (8, 14).

For cells which experience a transient isotopic disequilibrium during steady state photosynthesis, the SA at the intracellular site of carboxylation (SA_i) depends upon the SA of the DIC species which permeates the cells. Since the ratio of $^{14}C/^{12}C$ fixation depends upon the value of SA_i, the shape of a ^{14}C incorporation time-course is characteristic of the species of DIC taken by the cells (8, 14).

Theoretical time-courses of ^{14}C incorporation were calculated as described previously (8) for the situation in which only CO_2 or HCO_3^- is taken up for photosynthetic fixation by the cells. The value of the apparent rate constant of isotopic equilibrium (*i.e.* α_{ob}; 8) was estimated by nonlinear regression analysis using average rates of ^{14}C incorporation, determined from time-course experiments, and time as input variables (8). Calculations were performed by computer using the NLIN procedure (Gauss-Newton) of the Statistical Analysis System package (SAS Institute, Cary, NC). A natural logarithmic transformation of equation 25 (8) was employed as the nonlinear regression model and theoretical values of the apparent rate constant (*i.e.* α_1; Ref. 8) and the equilibrium rate of ^{14}C incorporation were used as starting values for the computer program. The theoretical values were determined as described in Espie and Colman (8).

RESULTS

Optimum Experimental Conditions. The quantity of $H^{14}CO_3^-$ or $^{14}CO_2$ introduced into the cell suspension to initiate isotopic disequilibrium is critical in that it should not significantly alter the bulk DIC concentration or cause an increase in the steady state rate of photosynthesis (8, 14). From a practical point of view, it is desirable that the volume of cell suspension sampled is small, to permit rapid and uniform termination of the reaction, while still containing a quantity of radioactivity which can be easily and accurately measured. We have, therefore, conducted preliminary experiments to determine the appropriate quantity of ^{14}C to add to the cell suspensions and to investigate the consequences of violating the criteria that have been outlined here.

Figures 1 and 2 are examples of the results of such experiments, when the DIC concentration (subsaturating for photosynthesis)

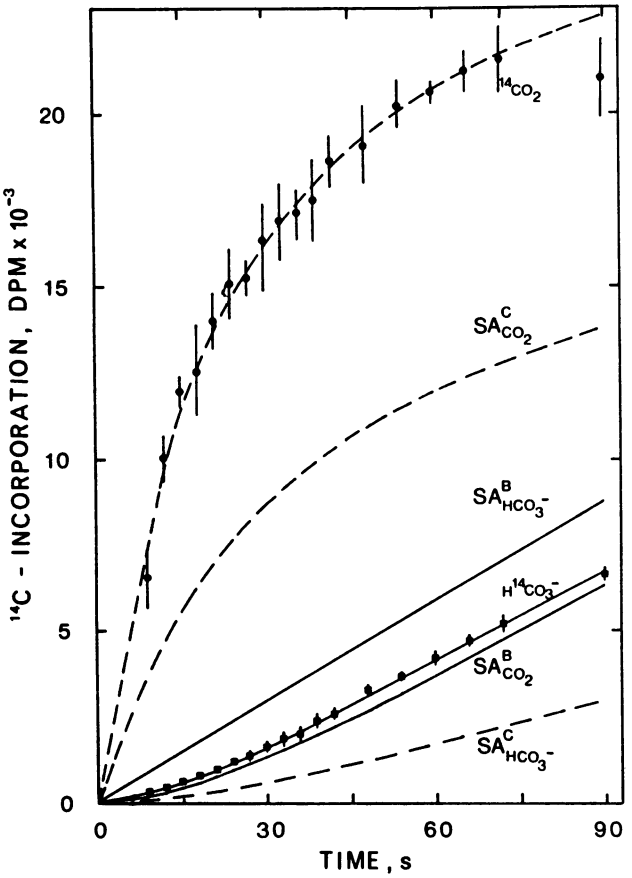

FIG. 1. Observed time-course of ^{14}C incorporation following the addition of $H^{14}CO_3^-$ (■) or $^{14}CO_2$ (●). *Asparagus* cells were allowed to establish a constant rate of O_2 evolution in buffered media (50 mM Hepes, pH 7.55), containing 269 μM DIC. Isotopic disequilibrium was initiated, at 25°C, by the addition of $H^{14}CO_3^-$ or $^{14}CO_2$, which represented a 6% increase in DIC concentration. Results are the average of triplicate determinations ± SD. Also shown are the theoretical time-courses of ^{14}C incorporation, following $H^{14}CO_3^-$ addition (——) or $^{14}CO_2$ addition (– – –), expected if SA_i equals the calculated SA of HCO_3 or CO_2 (as labeled). The calculations are based on the rate of photosynthesis (O_2 evolution) obtained just prior to ^{14}C addition.

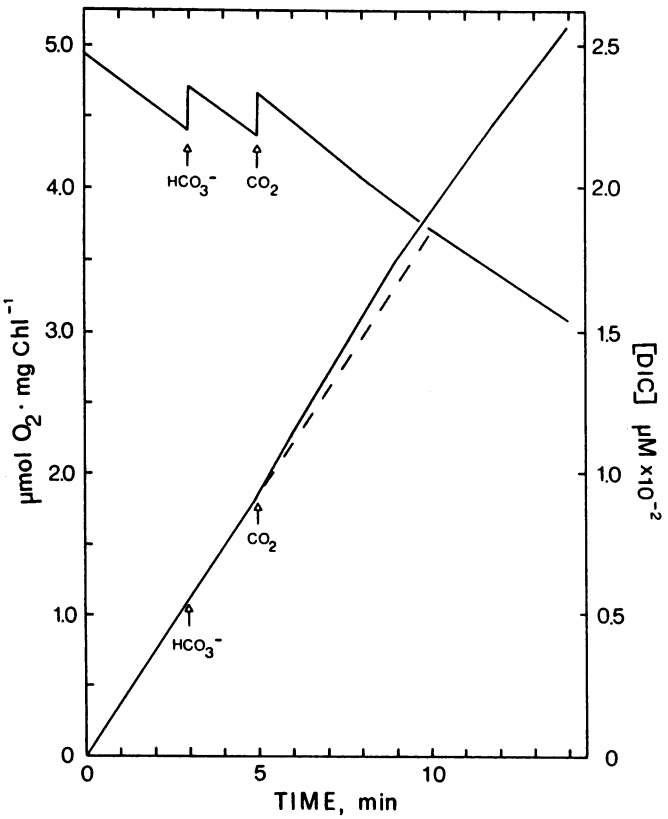

FIG. 2. Typical time-course of O_2 evolution (lower lines) following the addition of HCO_3^- or CO_2, calculated to yield a 6.1% increase in DIC concentration. Prior to the additions, *Asparagus* cells were allowed to establish a constant steady state rate of O_2 evolution in buffered media (50 mM Hepes, pH 7.5) containing an initial DIC concentration of 247 μM. The quantity of O_2 evolved was determined from the recorder trace of the O_2-electrode. The dashed line (- - -) represents the projected time-course of O_2 evolution following HCO_3^- addition. Also shown is the change in DIC concentration (upper line).

is increased 6% by the addition of $H^{14}CO_3^-$ or $^{14}CO_2$. Qualitatively, the form of the ^{14}C fixation time-course ($H^{14}CO_3^-$ addition; Fig. 1) is very similar to that expected when CO_2 serves as the sole source of DIC taken up by the cells, but the quantity of ^{14}C-fixed is slightly higher than predicted. The incorporation of ^{14}C is significantly higher than predicted when $^{14}CO_2$ is used to initiate isotopic disequilibrium. Again, however, the form of the ^{14}C fixation time-course is consistent with CO_2 being the major species of DIC taken up by the cells (Fig. 1). Theoretical considerations (8) indicate that ^{14}C incorporation in excess of the maximum possible incorporation, arising from CO_2 uptake, signifies a direct contribution of HCO_3^- ions to the supply of intracellular DIC. Interpretation of Figure 1 in this respect must, however, be tempered in light of the results shown in Figure 2.

In this experiment, O_2 evolution rather than ^{14}C fixation is monitored following the addition of HCO_3^- or CO_2. The results (Fig. 2) clearly show that the addition of CO_2, at this concentration, causes a significant, transient increase (approximately 30%) in the rate of O_2 evolution, while the addition of HCO_3^- has only a small effect. The transient stimulation of photosynthesis by the addition of CO_2 is not totally unexpected. Although the added CO_2 represents only a 6% increase in the DIC concentration, at the pH (7.5) of the cell suspension, this addition causes an initial (t = 0) 86% increase in the concentration of CO_2. Consequently, most or all of the excess ^{14}C incorporated (Fig. 1) can be attributed to the enhanced rate of photosynthesis rather than to the uptake of HCO_3^-.

We have found that an initial 1 to 2% increase in CO_2 or HCO_3^- concentration, as $^{14}CO_2$ or $H^{14}CO_3^-$, does not produce a detectable change in the rate of photosynthesis (O_2 evolution) and that good quantitative agreement between observed and predicted (CO_2 uptake) time-courses of ^{14}C incorporation is obtained at subsaturating DIC concentrations (9). However, to eliminate any possible ambiguity, we have conducted most of the subsequent experiments at DIC concentrations which are saturating for photosynthesis. Consequently, the rate of photosynthesis is independent of the added ^{14}C.

Inorganic C Uptake during Isotopic Disequilibrium. Figure 3A shows the time-course of ^{14}C incorporation into acid-stable products of photosynthesis by *Asparagus* cells, following the addition of a small quantity of highly labeled $H^{14}CO_3^-$ or $^{14}CO_2$ (55 μCi μmol^{-1}). Prior to the addition of ^{14}C, the cells were allowed to establish a constant rate of photosynthesis (43.2 μmol O_2 mg^{-1} Chl h^{-1} $\pm$ 1.5 SD, n = 12). This rate together with the calculated SA_{CO2} or SA_{HCO3}^- (8) was used to calculate the theoretical time-course of ^{14}C incorporation, when either CO_2 or HCO_3^- serves as the sole source of exogenous DIC taken up for photosynthetic fixation (Fig. 3B).

The addition of $H^{14}CO_3^-$ to the cell suspension results in a biphasic time-course of ^{14}C-fixation (Fig. 3A). A distinct lag in the incorporation of ^{14}C is observed, lasting 35 to 40 s, followed thereafter by an essentially constant rate of ^{14}C incorporation. A biphasic time-course is also observed when $^{14}CO_2$ is used to initiate isotopic disequilibrium. In this case, however, an initial rapid incorporation of ^{14}C is observed, followed by a much reduced but constant rate of ^{14}C incorporation, 35 to 40 s later.

The addition of CA, a potent catalyst of the CO_2-HCO_3^- interconversion reaction, to the cell suspension has a radical effect upon the pattern of ^{14}C incorporation (Fig. 3A). Linear instead of biphasic time-courses are observed in both instances. In the presence of CA, the constancy of the rate of photosynthesis (O_2 evolution) is immediately reflected in the rate of ^{14}C incorporation rather than after a 35 to 40 s delay. These results establish that the biphasic nature of the time-courses (−CA) are due to the slowness of the extracellular interconversion between CO_2 and HCO_3^-. Furthermore, these results indicate the absence of an effective extracellular catalyst of the CO_2-HCO_3^- interconversion reaction at the surface of the cells.

The shapes of observed and predicted time-courses (−CA), calculated on the basis of CO_2 uptake alone, are quite similar (Fig. 3, A and B). Quantitative agreement between the observed and predicted time-courses (CO_2 uptake) is also very good, particularly in light of the large number of factors required to make the quantitative predictions. These results, therefore, indicate that ^{14}C fixation by *Asparagus* cells occurs from an intracellular DIC pool in which the value of SA_i is closely approximated by the calculated value of SA_{CO2} rather than SA_{HCO3}^-.

Effect of pH. The effect of pH on the observed time-courses of ^{14}C incorporation is shown in Figure 4. Following the addition of $H^{14}CO_3^-$, no trace of inflection is evident in the ^{14}C fixation time-course (Fig. 4A) when the pH of the medium is 5.25. However, as the pH of the reaction medium is increased to 7.5 (Fig. 4, B–D), a distinct lag in the incorporation of ^{14}C arises. In all cases, very good quantitative agreement between experimental and predicted (CO_2 uptake) time-courses is found. Visual inspection of the time-courses does not reveal any significant deviation in the duration or degree of curvature, from that predicted when intracellular DIC is derived from the uptake of CO_2 alone. A more quantitative assessment of the curvature, however, can be obtained through analysis of the rate of ^{14}C incorporation by nonlinear regression (8).

If the kinetics of the CO_2-HCO_3^- interconversion reactions strictly control the rate of ^{14}C incorporation, then the observed rates will be exactly described by equation 25 (8), when CO_2

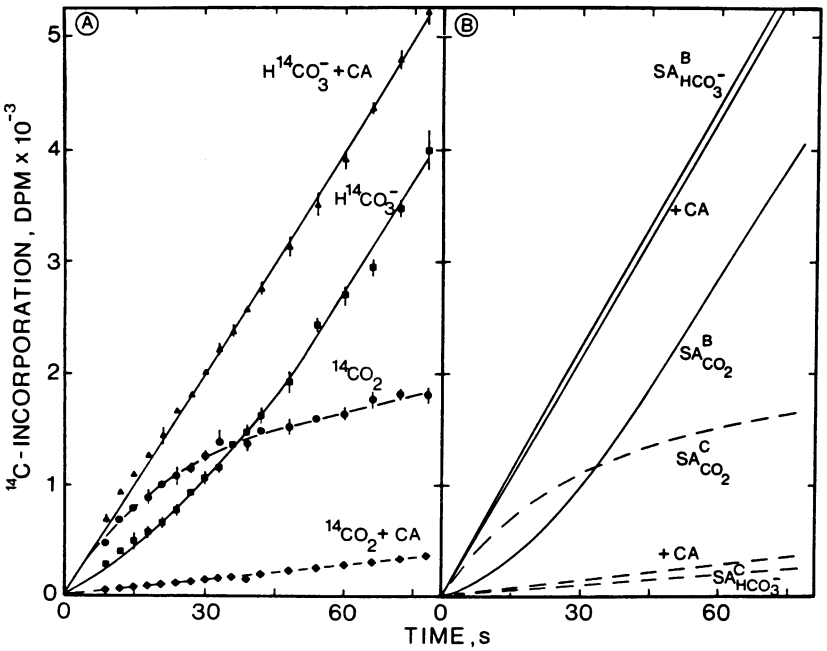

FIG. 3. A, Observed time-courses of ^{14}C incorporation following the initiation of isotopic disequilibrium by the addition of $H^{14}CO_3^-$ (■, ▲) or $^{14}CO_2$ (●, ♦) to cell suspensions containing + (▲, ♦) or − (■, ●) CA (0.1 mg·ml^{-1}) at 25°C. Results are the average of triplicate determinations ± SD. Prior to the addition, *Asparagus* cells were allowed to establish a constant rate of photosynthesis in 50 mM Hepes buffer (pH 7.49) containing 2.4 mM DIC. B, Theoretical time-courses of ^{14}C incorporation, following the addition of $H^{14}CO_3^-$ (——) or $^{14}CO_2$ (– – –), expected if SA_i equals the calculated $SA_{HCO_3^-}$ or SA_{CO_2} (as labeled) at all times during isotopic disequilibrium. Also shown are expected time-courses when CA (+CA) is included in the cell suspension and ^{14}C incorporation is calculated from the equilibrium (SA_{DIC}) SA.

alone is taken up by the cells. Estimation of the apparent kinetic constant (α_{ob}) of the interconversion reaction from time-course data can, therefore, provide a means to detect and assess the influence other factors may have on the uptake of DIC (8). Figure 5 shows the results of nonlinear regression analysis on the observed rate of ^{14}C incorporation as a function of time together with the expected rate of ^{14}C incorporation, if CO_2 or HCO_3^- alone is taken up by the cells. These data are derived from Figure 4. The nonlinear regression analysis indicates that the rate of ^{14}C incorporation is somewhat slower than predicted (CO_2 uptake) at acid pH (Fig. 5, B–C), but that close agreement is obtained between observed and predicted (CO_2 uptake) at a slightly alkaline pH (Fig. 5D). In these experiments, the values of α_{ob} are 0.069, 0.039, and 0.042 (Fig. 5, B–D, respectively) compared to theoretical values of α_1 of 0.120, 0.064, and 0.043 (Fig. 5, B–D).

Figure 5A is a special case in that sampling of the cell suspension was commenced (9 s) after 97% isotopic equilibrium had been attained. Consequently, the rate of ^{14}C incorporation reflects the constancy of the rate of photosynthesis at all times and, therefore, the data cannot be used to obtain an estimate of α_{ob}. However, linear regression analysis (Fig. 4A; $r = 0.9995$) indicates that the value of the extrapolated y-intercept is negative. This result is consistent with the occurrence of a small lag in the incorporation of ^{14}C prior to the sampling period, as predicted for CO_2 uptake.

Assessment of the Role of HCO_3^-. In experiments conducted at pH 8.5, a distinct lag in ^{14}C incorporation is also observed (Fig. 6A) which is consistent with the uptake of CO_2 (8, 14). In this case, however, ^{14}C incorporation exceeds the expected level and α_{ob} (Fig. 6B) is 1.6 times higher than the overall rate constant (α_1) of the uncatalyzed CO_2-HCO_3^- interconversion reaction. These results indicate that intracellular DIC is acquired from two external sources, CO_2 and HCO_3^- (8).

The role of HCO_3^- was further investigated in experiments in which the CO_2 concentration was held constant while the HCO_3^- concentration and pH were varied. At acid pH (6.0, Fig. 7) the calculated value of α_{ob} is 45% lower than the theoretical value (α_1), while at pH 7.5 this difference decreases to 15%. As CO_2 alone is taken up by the cells in the pH range 5.2 to 7.5 (Figs. 3–5), these observations indicate that the exchange flux of CO_2 between the medium and the cells is not instantaneous, but encounters some resistance, and that high HCO_3^- concentrations (at constant [CO_2]) enhances the velocity of the exchange flux (20).

In contrast, at pH 8.5, the measured value of the apparent rate constant exceeds the predicted value by 1.2- to 2.4-fold, indicating that HCO_3^-, as well as CO_2, permeates the plasmalemma. Using the equation given in Espie and Colman (8), we have calculated that the contribution of extracellular HCO_3^- to net DIC uptake is between 5 and 16%.

The relationship between HCO_3^- concentration and the uptake of HCO_3^- by *Asparagus* cells, at pH 8.5, is shown in Figure 8. In these experiments, the CO_2 concentration ranged from near the compensation point (6 μM) to saturation (150 μM). As indicated by the measured value of α (8), no evident dependence of HCO_3^- uptake on the extracellular HCO_3^- concentration occurs, although a direct contribution of HCO_3^- is indicated at all HCO_3^- concentrations examined (*i.e.* $\alpha_{ob} > \alpha_1$, Fig. 8). It is significant, however, that at HCO_3^- concentrations between 790 to 3000 μM (pH 8.5, Fig. 8) a direct contribution of HCO_3^- is indicated whereas at pH 7.5 and similar HCO_3^- concentrations (2500 μM, Fig. 5D; 250 μM, Ref. 9), HCO_3^- uptake is not indicated. This observation suggests that pH itself has a direct effect upon the cells, which alters the kinetics of ^{14}C incorporation.

DISCUSSION

The qualitative (shape) and quantitative (^{14}C incorporation) results of isotopic disequilibrium experiments indicate that CO_2, rather than HCO_3^-, is the primary source of exogenous DIC taken up for fixation by *Asparagus* cells during steady state photosynthesis (Figs. 3–5 and 7). The transient increase in the steady state rate of photosynthesis when *Asparagus* cells are challenged with a 6% increase in DIC concentration as CO_2, in contrast to the marginal effect of HCO_3^-, further supports this conclusion (Figs. 1 and 2).

Analysis of the rate of ^{14}C-incorporation, during isotopic disequilibrium (Figs. 5 and 7), indicates the presence of a small, extracellular, aqueous-phase resistance to the exchange flux of CO_2 between the cells and the medium. A similar conclusion has been arrived at by Volokita *et al.* (20) who found that at rate-limiting DIC concentrations for photosynthesis, exogenous CA stimulated the rate of *Asparagus* cell photosynthesis. This stim-

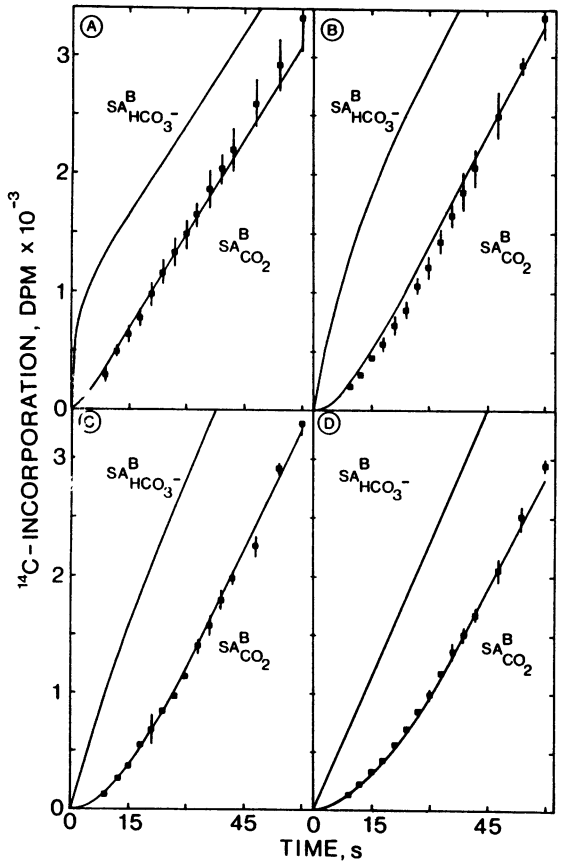

FIG. 4. Effect of pH upon observed (■) and predicted (——) time-courses of ^{14}C incorporation following the addition of $H^{14}CO_3^-$ to cell suspensions, photosynthesizing at a constant rate. Experimental conditions were: (A), pH 5.25; DIC = 218; PS = 31.2; (B), pH 6.00; DIC = 314; PS 42.4; (C), pH 6.50; DIC = 507; PS = 46.5; (D), pH 7.50; DIC 2712; PS 52.1. The unit of DIC concentration is μM and photosynthetic rate is in μmol $O_2 \cdot mg^{-1}Chl \cdot h^{-1}$. Data points are the average of triplicate determinations ± SD, except for (A) where $n = 6$. Solid lines represent the theoretical time-course of ^{14}C incorporation, expected if SA_i equals the $SA^B_{CO_2}$ or $SA^B_{HCO_3^-}$ (as labeled) at all times during isotopic disequilibrium.

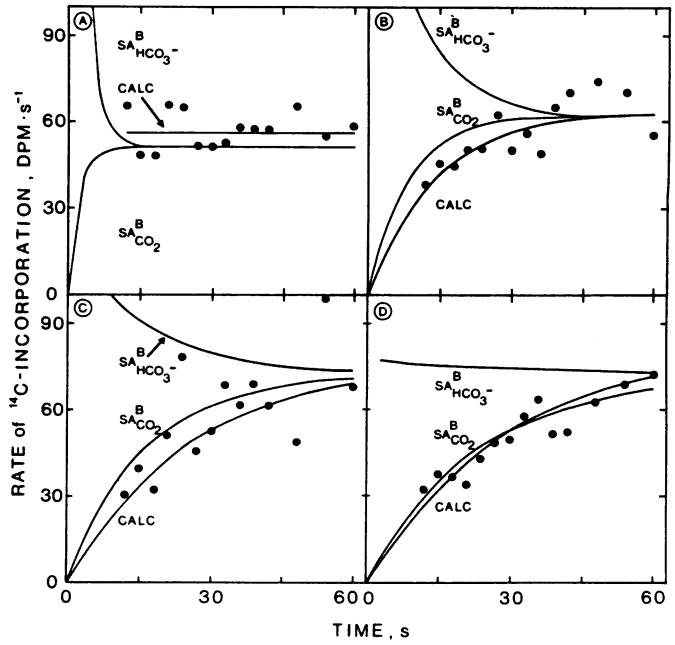

FIG. 5. Nonlinear regression analysis of the rate of ^{14}C incorporation as a function of time. The data are derived from Figure 4: (A), pH 5.25; (B), pH 6.00; (C), pH 6.50; (D), pH 7.50. The average rate of ^{14}C incorporation was determined, over small time intervals, as the slope of the line joining two consecutive data points, observed in time-course experiments. Shown are the theoretical rate of ^{14}C incorporation expected if SA_i equals either the $SA^B_{CO_2}$ or $SA^B_{HCO_3^-}$ (as labeled), and the results (CALC) of the nonlinear regression analysis.

ulation was attributed to the chemical facilitation by CA of CO_2 diffusion within the unstirred layer (10, 20). Apparently, the formation of CO_2 from HCO_3^- dehydration, in the absence of CA, was inadequate to alleviate the diffusion limitation. It is important to note that HCO_3^- uptake (with subsequent intracellular conversion to CO_2) would be expected to redress the rate limitation imposed by CO_2 diffusion. The significant stimulation of photosynthesis by CA (9, 20), therefore, suggests that the direct uptake of HCO_3^- is small. The results of experiments conducted at pH 8.5 (Figs. 6, 8) indicate that the contribution of HCO_3^- is, in fact, small representing from 5 to 16% of net DIC uptake over an HCO_3^- concentration range of 790 to 21,000 μM.

It has been suggested (19, 20) that the uptake of HCO_3^- by mesophyll cells occurs via a carrier-mediated process. Several experimental results presented here are inconsistent with this proposal. First, at similar HCO_3^- concentrations uptake of HCO_3^- is indicated at pH 8.5 (Figs. 6–8) but not at pH 7.5 (Figs. 3 and 5). Although the rate of transport might be expected to be affected by pH, total inhibition, in the presence of abundant substrate, seems unlikely. In *Anabaena variabilis*, HCO_3^- transport is not abolished at pH values as low as 6 (21). Second, the contribution of HCO_3^- to net DIC uptake would be expected to be dependent upon the HCO_3^- concentration, over the subsaturating range for photosynthesis. Instead, it is found that, although variable, the contribution of HCO_3^- is nearly a fixed proportion at all HCO_3^- concentrations examined (Fig. 8). It is possible that the HCO_3^- transport system is saturated even at the lowest HCO_3^- concentration used. This proposal implies that the K_m (HCO_3^-) of the transport system is lower than 800 μM and well below the half-saturation constant for photosynthesis ($K_{1/2}[HCO_3^-]$ = 3100 μM [pH 8.4]). In this event, the half-saturation constant of transport would be near the compensation concentration and, as net photosynthesis is zero, the HCO_3^- transport system would be an ineffective source of DIC for photosynthesis. If the transport system is saturated at low HCO_3^- concentrations, then the flux of HCO_3^- will be constant, and the fractional contribution of HCO_3^- to net DIC uptake should decline as the CO_2 concentration and rate of photosynthesis increases. This situation is also not observed (Fig. 8).

Alternative mechanisms to explain the uptake of HCO_3^-, other than carrier-mediation, have been discussed in Volokita *et al.* (20). To these alternatives we add the following. If the pH at the cell surface is more alkaline than the bulk medium, isotopic equilibrium will be attained more rapidly than predicted, resulting in SA_{CO2} being higher in value throughout the duration of the experiment. In order to rationalize the results of the experiments at pH 8.5 in terms of CO_2 uptake, the pH at the cell surface would have to be between 8.6 and 9.2.

Studies with intact spinach chloroplasts indicate that the membrane is not totally impermeable to HCO_3^- (11). At pH 8.5, the extracellular concentration of HCO_3^- is 3 to 4 times higher than the intracellular concentration of HCO_3^- (6), whereas at lower pH, the direction of the HCO_3^- concentration gradient is reversed. At high pH, this concentration gradient in conjunction with a leaky membrane would permit the passage of some HCO_3^- across the plasmalemma. Mixing of $H^{14}CO_3^-$ with the intracellular pool would then result in an SA_i which is higher than SA_{CO2}.

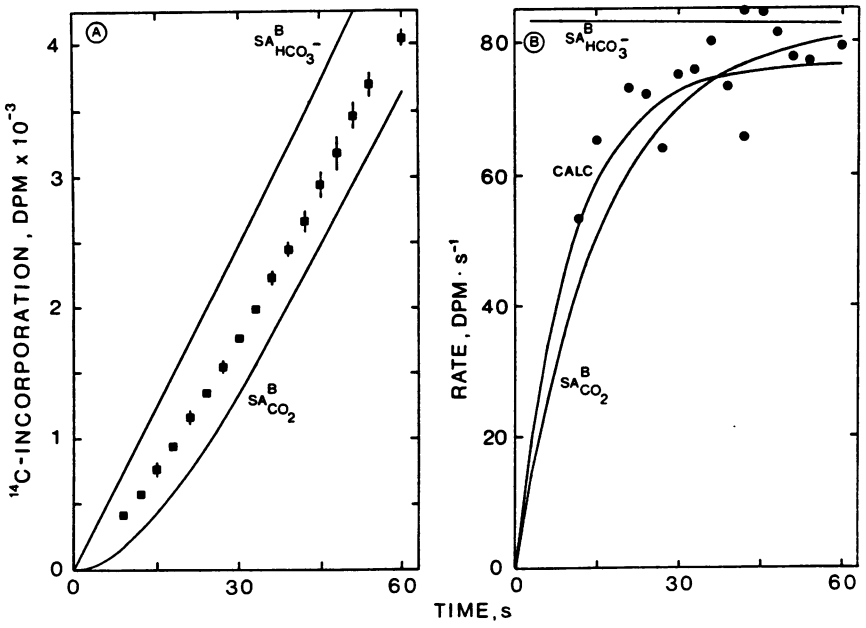

FIG. 6. A, Time-course of ^{14}C incorporation (■) following the addition of $H^{14}CO_3^-$ to a cell suspension photosynthesizing at a constant rate. Experimental conditions were: pH 8.45; DIC = 14,046 μM; PS = 35.9 μmol $O_2 \cdot mg^{-1}Chl \cdot h^{-1}$. Data points are the average of triplicate determinations ± SD. Solid lines represent the theoretical time-course of ^{14}C incorporation expected if SA_i equals $SA^B_{CO_2}$ or $SA^B_{HCO_3^-}$ at all times during isotopic disequilibrium. B, Nonlinear regression analysis of the rate of ^{14}C incorporation is a function of time. The data are derived from Figure 7A. Shown are the theoretical rate of ^{14}C incorporation expected if SA_i equal $SA^B_{CO_2}$ or $SA^B_{HCO_3^-}$ and the results (CALC) of the nonlinear regression analysis.

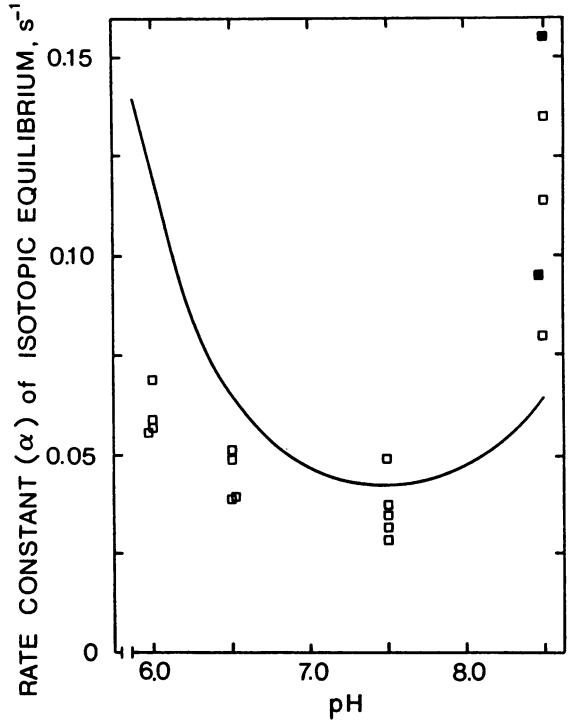

FIG. 7. Effect of increasing HCO_3^- concentration and pH on the value of α_{ob}. At each pH the DIC concentration was adjusted to yield a CO_2 concentration of approximately 170 to 200 μM CO_2 (□) or 100 to 110 μM CO_2 (■). Each data point represents the value of α_{ob} obtained from nonlinear regression analysis of triplicate time-course experiments. In all cases isotopic disequilibrium was initiated by the addition of $H^{14}CO_3^-$. Also shown (——) is the effect of pH on the theoretical value of α_i when CO_2 alone is taken up by the cells and no resistance to the exchange of CO_2 is assumed (*i.e.* $SA^B_{CO_2} = SA_i$).

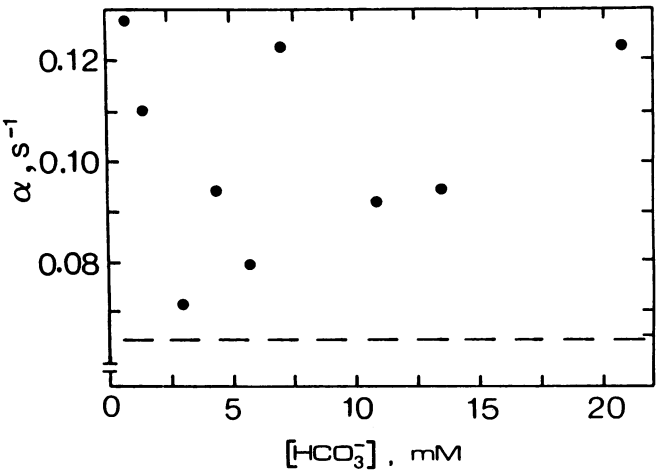

FIG. 8. Dependence of α_{ob} on the HCO_3^- concentration of the suspending medium (pH 8.5), and the expected dependence (---) when CO_2 alone is taken up by the cells and is in rapid equilibrium exchange (*i.e.* $SA^B_{CO_2} = SA_i$). Each data point represents the value of α_{ob} obtained from nonlinear regression analysis of duplicate time-course experiments. In all cases isotopic disequilibrium was initiated by the addition of $H^{14}CO_3^-$.

Although the data presented here and elsewhere (19, 20) indicate that *Asparagus* cells and pea protoplasts may take up HCO_3^- from the medium, the mechanistic basis is unclear. Previous studies with cyanobacteria (2, 9), organisms which transport HCO_3^- (13, 16), have shown that the patterns of ^{14}C incorporation, during isotopic disequilibrium, are quite different from those found for *Asparagus* cells. It seems unlikely, therefore, that both organisms utilize a similar mechanism for the uptake of DIC.

The pH of the extracellular fluid surrounding leaf mesophylls is presumed to be between 6 and 7 (17). Consequently, HCO_3^- uptake will not be a significant factor in the acquisition of DIC for photosynthesis *in vivo*.

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